

condition which allow primer-independent cleavage, the same quantities of molecules are placed in a solution that is the same as the buffer used in Reaction 1 regarding pH, enzyme stabilizers (*e.g.*, bovine serum albumin, nonionic detergents, gelatin) and reducing agents (*e.g.*, dithiothreitol, 2-mercaptoethanol) but that replaces any monovalent cation salt with 20 mM KCl; 20 mM KCl is the demonstrated optimum for primer-independent cleavage. Buffers for enzymes, such as DNAPEc1, that usually operate in the absence of salt are not supplemented to achieve this concentration. To test for primer-independent cleavage (Reaction 3) the same quantity of the test molecule, but no primer, are combined under the same buffer conditions used for Reaction 2.

All three test reactions are then exposed to enough of the enzyme that the molar ratio of enzyme to test complex is approximately 1:1. The reactions are incubated at a range of temperatures up to, but not exceeding, the temperature allowed by either the enzyme stability or the complex stability, whichever is lower, up to 80°C for enzymes from thermophiles, for a time sufficient to allow cleavage (10 to 60 minutes). The products of Reactions 1, 2 and 3 are resolved by denaturing polyacrylamide gel electrophoresis, and visualized by autoradiography or by a comparable method appropriate to the labeling system used. Additional labeling systems include chemiluminescence detection, silver or other stains, blotting and probing and the like. The presence of cleavage products is indicated by the presence of molecules which migrate at a lower molecular weight than does the uncleaved test structure. These cleavage products indicate that the candidate polymerase has structure-specific 5' nuclease activity.

To determine whether a modified DNA polymerase has substantially the same 5' nuclease activity as that of the native DNA polymerase, the results of the above-described tests are compared with the results obtained from these tests performed with the native DNA polymerase. By "substantially the same 5' nuclease activity" we mean that the modified polymerase and the native polymerase will both cleave test molecules in the same manner. It is not necessary that the modified polymerase cleave at the same rate as the native DNA polymerase.

Some enzymes or enzyme preparations may have other associated or contaminating activities that may be functional under the cleavage conditions described above and that may interfere with 5' nuclease detection. Reaction conditions can be modified in consideration of these other activities, to avoid destruction of the substrate, or other masking of the 5' nuclease cleavage and its products. For example, the DNA polymerase I of *E. coli* (Pol I), in addition to its polymerase and 5' nuclease activities, has a 3' exonuclease that can degrade DNA in a 3' to 5' direction. Consequently, when the molecule in Figure 16E is exposed to this polymerase under the conditions described above, the 3' exonuclease quickly removes the unpaired 3' arm, destroying the bifurcated structure required of a substrate for the 5' exonuclease cleavage and no cleavage is detected. The true ability of Pol I to cleave the structure can be revealed if the 3' exonuclease is inhibited by a change of conditions (*e.g.*, pH), mutation, or by addition of a competitor for the activity. Addition of 500 pmoles of a single-stranded competitor oligonucleotide, unrelated to the Figure 16E structure, to the cleavage reaction with Pol I effectively inhibits the digestion of the 3' arm of the Figure 16E structure without interfering with the 5' exonuclease release of the 5' arm. The concentration of the competitor is not critical, but should be high enough to occupy the 3' exonuclease for the duration of the reaction.

Similar destruction of the test molecule may be caused by contaminants in the candidate polymerase preparation. Several sets of the structure specific nuclease reactions may be performed to determine the purity of the candidate nuclease and to find the window between under and over exposure of the test molecule to the polymerase preparation being investigated.

The above described modified polymerases were tested for 5' nuclease activity as follows: Reaction 1 was performed in a buffer of 10 mM Tris-Cl, pH 8.5 at 20°C, 1.5 mM MgCl₂ and 50 mM KCl and in Reaction 2 the KCl concentration was reduced to 20 mM. In Reactions 1 and 2, 10 fmoles of the test substrate molecule shown in Figure 16E were combined with 1 pmole of the indicated primer and 0.5 to 1.0 µl of extract containing the modified polymerase (prepared as described above). This mixture was then incubated for 10 minutes at 55°C. For all of the mutant polymerases

tested these conditions were sufficient to give complete cleavage. When the molecule shown in Figure 16E was labeled at the 5' end, the released 5' fragment, 25 nucleotides long, was conveniently resolved on a 20% polyacrylamide gel (19:1 cross-linked) with 7 M urea in a buffer containing 45 mM Tris-borate pH 8.3, 1.4 mM EDTA. Clones 4C-F and 5B exhibited structure-specific cleavage comparable to that of the unmodified DNA polymerase. Additionally, clones 4E, 4F and 4G have the added ability to cleave DNA in the absence of a 3' arm as discussed above.

Representative cleavage reactions are shown in Figure 17.

For the reactions shown in Figure 17, the mutant polymerase clones 4E (*Taq* mutant) and 5B (*Tfl* mutant) were examined for their ability to cleave the hairpin substrate molecule shown in Figure 16E. The substrate molecule was labeled at the 5' terminus with ³²P. 10 fmoles of heat-denatured, end-labeled substrate DNA and 0.5 units of DNAP*Taq* (lane 1) or 0.5 μ l of 4e or 5b extract (Figure 17, lanes 2-7, extract was prepared as described above) were mixed together in a buffer containing 10 mM Tris-Cl, pH 8.5, 50 mM KCl and 1.5 mM MgCl₂. The final reaction volume was 10 μ l. Reactions shown in lanes 4 and 7 contain in addition 50 μ M of each dNTP. Reactions shown in lanes 3, 4, 6 and 7 contain 0.2 μ M of the primer oligonucleotide (complementary to the 3' arm of the substrate and shown in Figure 16E). Reactions were incubated at 55° C for 4 minutes. Reactions were stopped by the addition of 8 μ l of 95% formamide containing 20 mM EDTA and 0.05% marker dyes per 10 μ l reaction volume. Samples were then applied to 12% denaturing acrylamide gels. Following electrophoresis, the gels were autoradiographed. Figure 17 shows that clones 4E and 5B exhibit cleavage activity similar to that of the native DNAP*Taq*. Note that some cleavage occurs in these reactions in the absence of the primer. When long hairpin structure, such as the one used here (Figure 16E), are used in cleavage reactions performed in buffers containing 50 mM KCl a low level of primer-independent cleavage is seen. Higher concentrations of KCl suppress, but do not eliminate, this primer-independent cleavage under these conditions.